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Effects of different antimicrobials agents on mycoplasma species isolated from ruminants by macro culture technique

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Abstract

Mycoplasma is an important respiratory tract pathogen worldwide, causing respiratory tract infection in small ruminants (sheep and goats). It is a bacterium that causes acute respiratory illness ranging in severity from mild respiratory illness to severe pneumonia. Mycoplasmosis in small ruminants is a serious and major problem in Iran. This study was determined to isolate Mycoplasma species and detect antibacterial effect of Oxytetracycline, Tylosine, Chloramphenicole, Erythromycine, Enrofloxacin and Florfenocole on respiratory Mycoplasma subsp in small ruminantes by Macro culture technique. As, there is no an effective vaccine against Mycoplasma disease, treatment and controlling is mainly by chemotherapy. Basically, it was approved that uncontrolled usage of antimicrobial elements has caused the development of antimicrobial resistance. The antimicrobial susceptibility test showe some Mycoplsma species –specific differences, with *M.capricolum* subsp. It was more susceptible to erythromycine and Tylosine, while Florfeniclole and Chloramphenocole were the least effective for all three Mycoplasma species. It is observed that there was not any significant difference in antimicrobial susceptibility between goat and sheep isolates or between isolate from different regions in affected province. Results showed that some isolates of *M.capricolum*.and *M. putrefaciens* had minimal inhibitory concentration (MIC) level with Oxytetracyclin as was the same with two isolates of *M.mycoides* subsp. *mycoides LC* with Tylosine. It seems resistance factor against antimicrobials is involved.

Keywords: Antimicrobials, Mycoplasma, small ruminants, Macro culture

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Introduction

Mycoplasmas cause some of the most serious and economically significant diseases in livestock and pose major problems for animal health authorities worldwide. Infection has spread in the last five years to new regions and species, but little effective control is available, particularly in developing countries. Basically small ruminants are affected by several different Mycoplasma species, which cause a serious and significant impact like economic losses and problems influencing countries in Asia and Africa and Mediterrianean region. Mycoplasma species, is causative organism of contagious caprine pleuropeumonia. There are 60-70 million sheep and goats in Iran therefore, respiratory disease is considered to have a significant economic impact (Nicholas, *et al*., 2008).

During December 1999 to June 2000 in six cities in Fars province, disease caused by Mycoplasma agalactia was occurred. From 852 milk samples and ocular swabs, 10% showed positive reaction. The results indicated six *M.agalactia*,three M. mycoides subsp. and one M.capricolum subsp being isolated from sheep and goats (Ghaleh, 2006). 15.38% and 11.67 % of sheep and goats were infected respectively. *Mycoplasma agalactiae* and Mycoplasma subsp, mycoides strains were isolated from small ruminants (Aarabi and Sotoudehnia, 1984).

As there is not any potential vaccine for treatment, prevention and controlling of mycoplasmosis, therefore, selection of an effective antimicrobial agent is seemed to be very significant. Due to lack of cell walls in Mycoplasma strains of sheep and goats, antimicrobials as penicillin and cephalosporine are not applied because of inhibition of the cross – linking of amino acid chains in peptiddologycan synthesis. Mycoplasma species had resistance to sulphonamides group that inhibit synthesis of folic acid and those aminoglycosides that affect protein synthesis (Puglisi *et al*., 2000)

Mycoplasmas are more likely to be sensitive to tetracycline, which affect amino acid transfer to growing peptide chains at ribosome complexes. The main problem is the presence of resistant strain of mycoplasma to these and other generally effective antimicrobials which is well documented (Bebear, 1996). On the other hand, antimicrobial resistancein human caused by consumption of antimicrobials in treated sheep and goats is very important. In this survey, the activity of identified antimicroboials against *M.capricolum subsp, mycoides LC* and M.capricolum subsp Capricolum isolated from currently affected small ruminants was conducted.

Materials and methods

Nasal swabs from affected sheep and goats were collected for isolation of M. isolates and inoculated in Hella cells with Dulbeccos modified eagles medium (DMEM), enriched with 10% heat inactivated calf serum and 0.1% glucose under a standard condition (Nicholas *et al.*, 2000). After a few passage, Hella cells were treated with 0.02 ethylene diamin tetra acetic acid (EDTA) and 0.25% trypsin, then the last passage was freezed in maintenance medium culture accompany with 5% dimethyle sulphocide (DMSO) as a preservative and then maintained at -20°c for the next steps.

Mycoplasma isolates were identified by routine identification methods for mycoplasma including inhibition test (HI) by using mycoplasma–specific polyclonal rabbit antiserum (Poveda and Nicholas,1998) and molecular methods. Isolates were identified as *M. putrefaciens* and M. mycoides subsp. mycoides large colony type and M. capricolum subsp.capricolum by polymerase – chain reaction (PCR) under standard protocol (Bashiruddin *et al*.,1994). More details are given in Tables 1 and 2.

Preparation of cultures for antimicrobial susceptibilities

Mycoplasm isolates were grown in 4 - 6 ml of growth medium for 2-3 days in incubator at 37°c contained %5 Co_2. Standardization of inoculums was done by measuring the optical density (OD) of the broth culture at 450 nm. Concentration was adjusted to 0.1 with growth media that was approximately 10^8 colony forming units (CFU) per ml (Loria *et al*., 2003).

Table 1. Mycoplasma species isolated from sheep and goats in nasal swabs.

Species	Nasal swabs	Milk (sheep)	Nasal Swabs	Milk (Goats)
M. putrefaceiens	5	0	5	0
M. mycoides subsp	2	0	1	0
mycoides LC				
M. capricolum subsp.	2	0	1	0
Caprocolum				

Table 2. MIC values for each Mycoplasma isolates.

M.putrefaciens	**M.capricolum subsp**		
Antimicrobial	**Range**	**MIC_{50}**	**MIC_{90}**
	(µg /ml)	(µg /ml)	(µg /ml)
Oxytetracycline	0.25-1.00	0.25	1.00
Erythromycin	0.25-0.25	0.25	0. 25
Tylosin	0.12-0.12	0.12	0.12
Enrofloxacin	0.25-0.25	0.25	0.25
Flofenicol	0.50-4.00	1.00	4.00
Chloramphenic	1.oo-8.00	1.00	4.00
M. mycoides subsp. mycoidesLC			
Oxytetracycline	0.12-0.25	0.25	0.25
Erythromycin	0.25-0.25	0.25	0.25
Tylosin	0.06-1.00	0.06	1.00
Enrofloxacin	0.12-0.12	0.12	0.12
Flofenicol	2.00-4.00	2.0 0	4.00
Chloramphenic	8.00-8.00	8.00	8.00
M.capricolum subsp Capricolum			
Oxytetracycline	0.25-2.00	0.25	2.00
Erythromycin	<o.03- <03	<0.03	<0.03
Tylosin	<o.03- <03	<0.03	<0.03
Enrofloxacin	0.25-0.25	0.25	0.25
Flofenicol	4.00-8.00	4.00	8.00
Chloramphenic	8.00-8.00	8.00	8.00

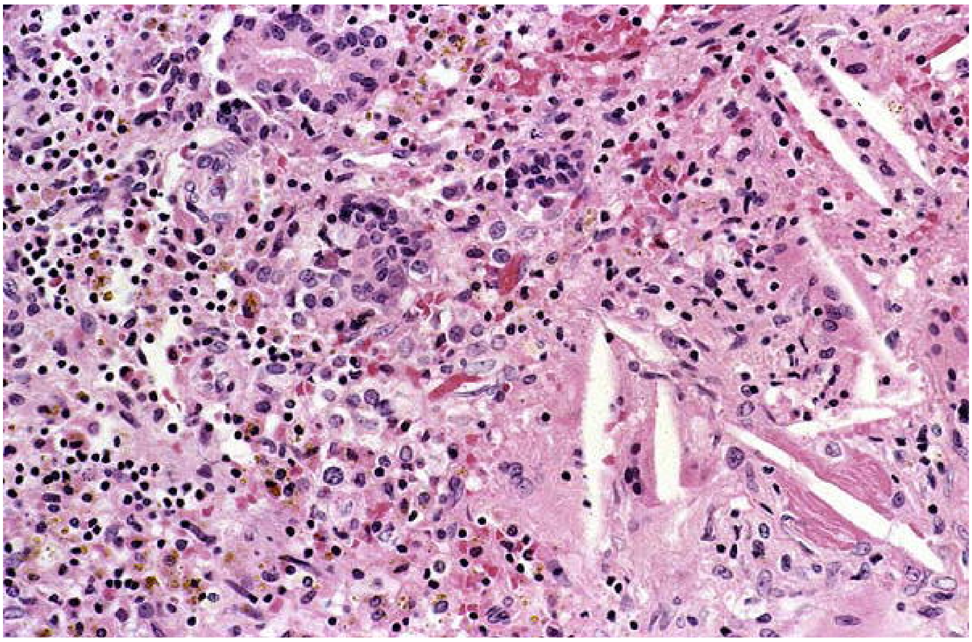


Figure1. *Mycoplasma agalactiae* in culture after a week.

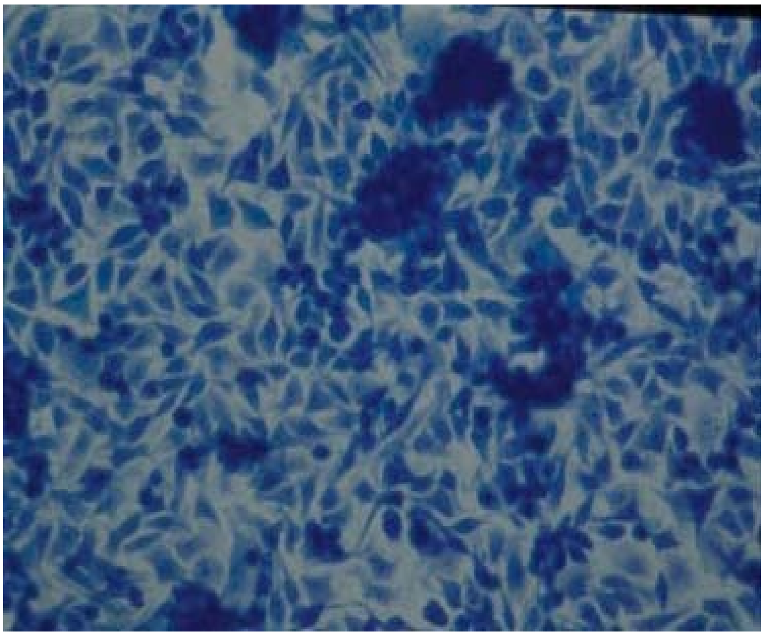


Figure 2. ***M.putrefaciens*** **replication after a few days.**

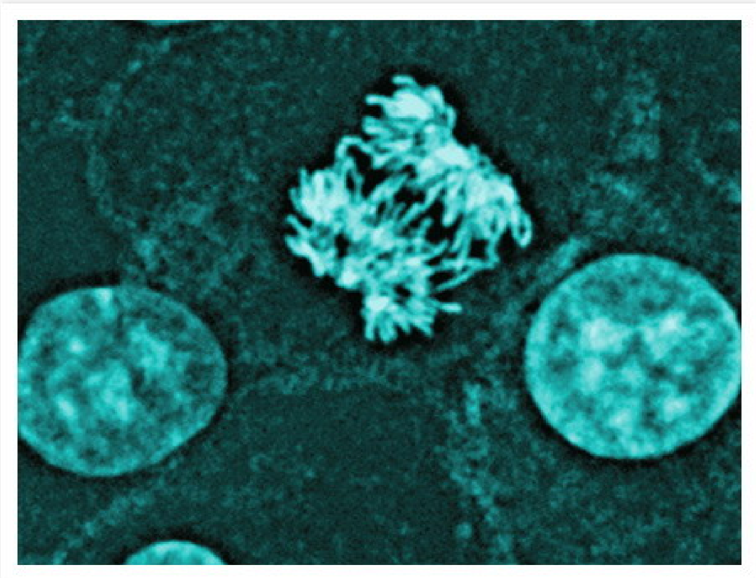


Figure 3. Colony forming units of ***mycoides LC in vitro.***

They were kept in 5-10 ml growth media of DMEM medium that was enriched with 10% fetal calf serum. Mycoplasma isolates were cultured and proliferated after remaining in 37 ^{0}c in 5% Co_2 incubator for two to three days which 450 nm optical density inoculums was established. The concentration was adjusted to 0.1with media that is equivalent to 10^8/ml CFU (Loria *et al.*, 2003).

Antimicrobial susceptibility test

Oxytetracycline, florfenicole,chloramphenicole, tylosin and erythromycine were used as antimicrobials,

according to NCCLS guidline (Woods and Washington,1995). Concentration of the antimicrobials were made to cover doubling dilution in range from 0.03-32 µg/ml.

Determination of minimum inhibitory concentration in vitro (MIC)

The minimum inhibitory concentration (MIC) was measured by *in vitro* method (Roberts, 1992), following the guidelines of Hanna, 2000. Tested group with final antimicrobial dilution of 0.03-32 µg /ml was compared with a control containing no antimicrobial.

Each well was filled with 80 µl with appropriate concentration of the selected antimicrobial and the control well was filled with 80 µl of double distilled water, then 300 µl of DMEM broth was added to all 96 wells that contained phenol red as indicator.The 20 µg of mycoplasma culture with 10^8 cfu /ml was added to all microtitre plate wells. Final concentration of mycoplasma in the wells was calculated. It was nearly to 5x 10^6 cfu /ml then, sealed plates were maintained in 37° C for two days. The mycoplasma MIC was defined according to the minimal concentration that inhibit color changes when control well was developed the color indicating for growth of Mycoplasma (Taylor *et al.,* 1997, Yamaguchi *et al*., 2000 and Ayling *et al.,* 2000)

Results

Mycoplasma species isolated *in vitro* culture from sheep and goats are shown in table 1, figures 1, 2, and 3. MIC range values between MIC_{50} to MIC_{90} and also M.strains for each tested antimicrobial are shown in table 2. These data indicate that the *in vitro* effectiveness of each tested antimicrobial varies depending on the Mycoplasma species. MIC values that were obtained in taylosin and erythromycin for M.putrifaciens and *M. mycoides* subsp *mycoides LC* were higher than those obtained for *M. capricolum* with taylosin and erythromycin. Also, a few isolates showed higher MIC values than other isolates of the same species against certain antimicrobials. For instance, *M.mycoides* subsp. mycoides LC had an MIC value of 1.00 µg /ml against tylosin compared to 0.06 µg /ml that was obtained for two isolates. MIC values of *M.putrefaciens* was 1.00 µg /ml for oxytetracycline in comparison with 0.25 µg /ml for other isolates and MIC values of M. capricolum species was 2.00 µg /ml against oxytetracycline. So. among mycoplasma species that were tested, only M.putrefaciens had wide ranging results via chloramphenicole (1-8 µg /ml) and florfenicol (0.5-4 µg /ml)

Discussion

Up to now,limited investigations were carried out about sensitivity and resistance of mycoplasma subsp against antimicrobials and also MIC value (Ayling *et al*., 2000, Loria *et al*., 2003). The results showed that antimicrobial sensitivity *in vitro,* is same as in vivo. Therefore, treatment of Mycoplasmosis is completely based on selection of an effective antimicrobial agent with high MIC values.

Although, in this survey a few isolates were tested but decision and prediction for treatment of macoplasmosis completely depends on tests on clinical strains.

Both erythromycin and tylosin were the most effective drugs in vitro, whereas enrofloxacin showed the least MIC values for *M. mycoides* subsp and *mycoides LC* and tylosin for *M. purtrefaciens*, however, isolation and specification of these pathogens would be carried out on the affected animals so the antimicrobials with the best result should be selected for treatment. It seems difficult to recommend just one effective antimicrobial *in vivo.* The descriptions intermediate, resistant or sensitive are routine to all methods of clinical laboratory testing, and are distinguished by in vitro breakpoint antimicrobial concentrations. Pharmacological and microbiological factors are two important factors in determining breakpoints. These factors have not been determined for mycoplasmas in veterinary

literature. Hannan (2000) worked on different mycoplasma species and reported that tylosin, erythromycin and enrofloxacin should be less than 1 µg/ml and oxytetracyclin and chlroamphenicol less than 4 µg/ml so, with these data, erythromycin, enrofloxacin and oxytetracycline are choice drugs and MIC values of oxytetracycline are near to the breakpoint, and regarding to the developing resistance, it might be avoided. We concluded that antimicrobials with high MIC value did not necessarily result in higher MIC values in comparison to other antimicrobials, even between the paired antimicrobials as tylosin and erythromycin. In this survey, these three Mycoplasmas were obtained form both goats and sheep. In small ruminants, often a variety of antimicrobials are used for disease without veterinary supervisions and this may lead to disease recurrence later with more severe symptoms. On the other hand, uncontrolled usage of antimicrobials can cause a developing antimicrobial resistance against mycoplasm species.The results showed some species with higher MIC values than others with species-specific differences, for instance, *M. capricolum* subsp is more susceptible to erythromycin and tylosin. Florfenicole and chloramphenicole had the least effectiveness against all three species. It is noted that there is not any significant difference in antimicrobial susceptibility in sheep and goat isolates. Some isolates of mycoplasma species were isolated from sheep and goats in nasal swabs. *M. capricolum* and *M. putrefaciens* had higher MIC values with oxytetracycline which was the same as two isolates of *M.mycoides* subsp, *mycoides LC* for tylosin, which representing the development of antimicrobial resistance (Hannan, 2000).

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ارزیابی اثر آنتی‌باکتریال‌ها بر جدایه‌های مایکوپلاسمای دامی با استفاده از تکنیک کشت ماکرو

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چکیده

مایکوپلاسما یکی از شایعترین عوامل بیماریزای مجرای تنفسی است که باعث عفونت سیستم تنفسی در نشخوار کنندگان کوچک (گوسفند و بز) می‌شود. این باکتریئ موجب بیماری پنومونی به شکل ملایم و یا شدید شده و در حال حاضر مایکوپلاسموز در نشخوارکنندگان کوچک در ایران یک مشکل اساسی است. این مطالعه به منظور جداسازی جدایه های مایکو پلاسما و شناسائی اثر ضد باکتریائی اکسی تتراسیکلین ، تایلوزین ، کلرامفنیکل، اریترومایسین، انرو فلوکسازین و فلورفنیکل بر برخی از جدایه‌های تحت گونه مایکوپلاسمای جدا شده از نشخوارکنندگان کوچک با استفاده از تکنیک کشت ماکرو انجام شد. از آنجائی که واکسن موثری بر علیه این بیماری وجود ندارد، درمان و کنترل آن با استفاده از درمان داروئی انجام می‌شود. اصولاً مصرف بی‌رویه آنتی بیوتیک‌ها سبب مقاومت داروئی می‌شود. آزمایش حساسیت آنتی باکتریال نشان داد که در برخی از جدایه‌های مایکوپلاسما مانند، مایکوپلاسما کاپریکولوم بیشترین حساسیت نسبت به اریترومایسین و تایلوزین و کمترین حساسیت نسبت به کلرامفنیکل و فلورفنیکل وجود دارد. هیچگونه تفاوتی در حساسیت‌های آنتی باکتریالی در جدایه‌های گوسفند و بز در نواحی مختلف استان‌های در گیر بیماری مشاهده نگردید. نتایج نشان داد که برخی از جدایه‌های مایکوپلاسما کاپریکولوم و مایکوپلاسما پوتریفیکانس دارای بیشترین میزان بازدارندگی در اکسی تتراسیکلین و دو جدایه تحت گونه مایکوپلاسما میکوئیدس و میکوئیدس ال. سی دارای حساسیت همسانی در برابر تایلوزین هستند.

واژگان کلیدی: آنتی باکتریال، مایکو پلاسما، نشخوارکنندگان کوچک، کشت ماکرو